Chem. Pharm. Bull. 30(3)1063—1065(1982)

Studies on LM Protein appearing in Submandibular Glands of Isoproterenol-treated Rats. IV. Size and Shape Determination

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(Received August 5, 1981)

Some physicochemical and biochemical properties of the purified large mobile (LM) protein appearing in submandibular glands of isoproterenol-treated rats were studied. The molecular weight of this protein was found by sedimentation equilibrium and gel chromatography on Sepharose 6B to be 12700—13000. The partial specific volume was estimated to be 0.72 and the Stokes radius to be 28.8 Å. The frictional ratio of the LM protein was found to be 1.69 by viscometry by assuming that the hydration of the protein is zero and adopting a prolate ellipsoidal model. The circular dichroism spectra of the LM protein showed that most of the peptide chain took random coil (64%) and β-structure (30%) conformations. These results suggest that the LM protein has a slightly elongated shape. The LM protein did not show measurable activities of deoxyribonuclease, ribonuclease, lysozyme, nerve growth factor or phospholipase.

Keywords——submandibular gland; isoproterenol; sedimentation equilibrium; viscometry; circular dichroism

Introduction

Menaker *et al.*[1] first reported that a large mobile (LM) protein appeared in the enlarged submandibular glands of isoproterenol (IPR)-treated rats. We have purified the LM protein, and the mechanism of its secretion through the action of β-adrenergic receptors has been partly clarified.[2] Some physicochemical properties such as molecular weight (MW), isoelectric point, sugar content, calcium content, amino acid composition and a part of the amino acid sequence from the N-terminal of the LM protein were also reported.[3] This communication reports further characterization of the physicochemical properties of LM protein. Despite various attempts, we failed to find any biochemical activities of the protein.

Materials and Methods

Isolation of LM protein from IPR-treated rat submandibular saliva was reported previously.[2] Molecular weight determination by gel chromatography on Sepharose 6B was carried out according to the method of Fish *et al.*[4] The distribution coefficient (K_{av}), defined as $K_{av}=(V_e-V_o)/(V_i-V_o)$, on Sepharose 6B column (1.2×51 cm) chromatography was measured by the use of blue dextran 2000 (Pharmacia). Bromophenol blue (B.P.B) was used as a marker for the internal volume (V_i) of the column, and V_e denotes the elution volume of the protein. The proteins used as standards were bovine serum albumin (MW 66000), α-chymotrypsin (MW 21600) (Sigma), catalase (MW 40000), cytochrome C (MW 13370) (Boehringer Manheim) and insulin (MW 5700) (Fluka AG). Molecular weight was also determined by the Yphantis sedimentation equilibrium method[5] with a Centriscan 75 analytical ultracentrifuge (MSE). The partial specific volume ($\bar{V}$) of the LM protein was calculated from its amino acid composition[3] according to the method of McMeekin.[6] Stokes radius determination was performed by gel chromatography on Sepharose 6B as described above. The frictional ratio was determined by using Simha's equation[7] and Perrin's function[8] with an Ostwald microviscometer. Secondary structure was determined by measuring the circular dichroism spectra of LM protein dissolved at a concentration of 0.218 mg/ml in 0.01 M phosphate buffer (pH 7.2) at 20°C (JASCO-20 automatic recording spectropolarimeter). α-Helix content was estimated according to the method of Chen and Yang,[9] and β-structure and random coil contents by the method of Greenfield and Fasman[10] using the spectra of synthetic poly (L-lysine).

Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard. Deoxyribonuclease activity[12] and ribonuclease activity[13] were measured by the procedure of Kunitz. Lysozyme activity was assayed according to the method of Shugar[14] with a substrate from *M. luteus*. Activity as nerve growth factor[15] was kindly assayed by Dr. Hiroshi Saitoh, Faculty of Pharmaceutical Sciences, the University of Tokyo, Tokyo. Phospholipase activity was examined in the presence or absence of 1 mM $CaCl_2$ by using egg lecithin as a substrate. Other enzyme activities were assayed by the clinical test system used in Daiyūkai Hospital, Ichinomiya, Aichi.

Results and Discussion

Molecular weight was determined by gel chromatography on Sepharose 6B in guanidine hydrochloride. The calibration curve of molecular weight (a semi-logarithmic plot of molecular weight *vs* K_{av}) showed a good straight line. Irrespective of the absence or presence of 2-mercaptoethanol in the solution, the value of K_{av} of the LM protein was 0.42, and the elution pattern was unchanged. From these results, the molecular weight of LM protein was calculated to be 13000 (data not shown). The molecular weight was determined by sedimentation equilibrium analysis. A linear relationship of $-\ln A_{280\,nm}$ *vs* r^2 was obtained, indicating a monodisperse system (data not shown). The slope $d(-\ln A_{280\,nm})/dr^2$ was calculated to be 0.466. The partial specific volume ($\bar{V}$) of the LM protein was calculated from its amoin acid composition[3] to be 0.72 ml/g. From these values, the molecular weight of LM protein was evaluated to be 12700 by means of the equation $MW=2\boldsymbol{R}T/(1-\bar{V}\rho)\ \omega^2\times d(-\ln A_{280\,nm})/dr^2$. In this equation, $\boldsymbol{R}$, T, ω, ρ and r stand for the gas constant, the absolute temperature, the angular velocity, the density of the solvent and radius, respectively. The Stokes radius of the LM protein was estimated by gel chromatography on Sepharose 6B as shown in Fig. 1. According to Laurent and Killander's equation[16]; $(-\log K_{av})^{1/2}=\alpha(\beta+\gamma)$, the square root of the logarithm of $1/K_{av}$ and Stokes radius were plotted on the ordinate and abscissa, respectively. A linear relationship of $(-\log K_{av})^{1/2}$ *vs* Stokes radius was obtained. The K_{av} of LM protein was less than that of α-chymotrypsin and its Stokes radius was estimated to be 28.8 Å. The frictional ratio was determined by viscometry. The reduced viscosity was plotted against the protein concentration (data not shown). The intrinsic viscosity of this protein was estimated to be 14 ml/mg. This value corresponds to a volume fraction limiting viscosity number of 19.4 ml/ml. The axial ratio of this protein was calculated to be 12.9 from Simha's equation.[7] For calculation of the frictional ratio (f/f_0) of this protein, this value was applied to Perrin's function[8] to obtain a value of 1.69. On the other hand, f/f_0 was also calculated from the MW obtained by sedimentation equilibrium and the Stokes radius obtained by gel chromatography on Sepharose 6B, using the equation $f/f_0=S/(3\bar{V}\cdot MW/4\pi\cdot N)^{1/3}$ where N is Avogadro's number and S is the Stokes radius. f/f_0 of the LM protein was calculated to be 1.86. The value is in fairly good agreement with that obtained by viscometry. Secondary structure was determined from the circular dichroism spectrum. The value of $(\theta)_{208}$ (-5825 deg·cm²/d mol) was inserted into Chen and Yang's equation, α-helix content $=(\theta)_{208}-(-4000)/-33000-(-4000)$, to obtain an α-helix content of 6%. The contents of β-structure and random coil were estimated to be about 64% and 30%, respectively (data not shown). An acidic proline-

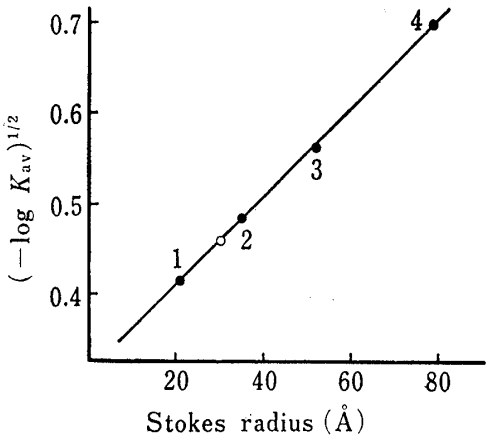

Fig. 1. Stokes Radius Determination by Gel Chromatography on Sepharose 6B

A calibration curve of $(-\log K_{av})$ *vs* Stokes radius was obtained as described in the text. The LM protein is represented by ○. The standard proteins (●) have Stokes raidii (Å) of 1, α-chymotrypsin (21); 2, bovine serum albumin (35); 3, catalase (52); 4, ferritin (79).

Table I. Comparison of the Physical Properties of the LM Protein and Acidic Proline-rich Protein

	LM protein	Acidic proline-rich Protein[a]
$s_{20,w}$		1.4
Molecular weight	12000—13000	24500
Stokes radius (Å)	28.8	46±8
Axial ratio	12.9	25
f/f_0	1.69	2.3

a) Results reported by Muenzer *et al.*[18]

rich protein was found in parotid glands of rats after chronic administration of IPR,[17,18] and some of its physicochemical properties were analyzed by Muenzer *et al.*[18] Some properties of the LM protein and the acidic prolineerich protein are compared in Table I. Although the shape of LM protein was similar to that of acidic proline-rich protein, there are many differences in the physicochemical properties of those proteins, indicating that these proteins are different.

The LM protein did not show measurable activities in assays for deoxyribonuclease, ribonuclease, lysozyme, nerve growth factor and phospholipase activities. Other enzyme activities tested by means of our clinical test system were leucine aminopeptidase, glutamate pyruvate transaminase, glutamate oxaloacetic transaminase, amylase, alkaline phosphatase, acid phosphatase, lactate dehydrogenase, monoamine oxidase and cholinesterase. No measurable activities were detected in these assays. Further efforts are necessary to identify the cause and the effect of the secretion of LM protein after IPR stimulation.

Acknowledgement We wish to thank Assoc. Prof. Hirosi Saitoh, Faculty of Pharmaceutical Sciences, the University of Tokyo, Tokyo, for kindly performing assays for nerve growth factor, and also Prof. Harumi Okuyama, the Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya, for his help in preparing the manuscript.

References and Notes

1) L. Menaker, J.H. Sheetz, C.M. Cobb, and J.M. Navia, *Lab. Invest.*, **30**, 341 (1974).
2) Y. Naito, *Chem. Pharm. Bull.*, **29**, 1365 (1981).
3) Y. Naito and I. Suzuki, *Chem. Pharm. Bull.*, **29**, 1373 (1981).
4) W.W. Fish, K.G. Mann, and C. Tanford, *J. Biol. Chem.*, **244**, 4989 (1969).
5) D.A. Yphantis, *Biochemistry*, **3**, 297 (1964).
6) T.L. McMeekin, M.L. Groves, and N.J. Hipp, *J. Am. Chem. Soc.*, **71**, 3298 (1949).
7) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).
8) P.F. Perrin, *J. de Phy.*, **7**, 1 (1936).
9) Y.H. Chen and J.T. Yang, *Biochem. Biophys. Res. Commun.*, **44**, 1285 (1971).
10) N. Greenfield and G.D. Fasman, *Biochemistry*, **8**, 4108 (1969).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) M. Kunitz, *J. Gen. Physiol.*, **33**, 349 (1950).
13) M. Kunitz, *J. Biol. Chem.*, **164**, 563 (1946).
14) D. Shugar, *Biochim. Biophys. Acta*, **8**, 302 (1952).
15) E.L. Fenton, *Exp. Cell Res.*, **59**, 383 (1970).
16) T.C. Laurent and J. Killander, *J. Chromatog.*, **14**, 317 (1964).
17) A. Fernandez-Soresen and D.M. Carlson, *Biochem. Biophys. Res. Commun.*, **60**, 249 (1974).
18) J. Muenzer, C. Bildstein, M. Gleason, and D.M. Carlson, *J. Biol. Chem.*, **254**, 5629 (1979).